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Changes in Subsurface Catabolic Gene Frequencies during Natural Attenuation of of Petroleum Hydrocarbons

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Subsurface hydrocarbon contamination associated with petroleum spills is a widespread occurrence that presents a significant threat to groundwater resources. The natural attenuation test site (NATS) at Columbus Air Force Base, MS, provided a unique opportunity to monitor changes in the molecular microbial ecology as well as stimulation of natural biodegradative processes under transient field study conditions. A large, synthetic jet fuel mixture containing BTEX compounds and naphthalene in a decane carrier was introduced into the subsurface. Over 462 days, a plume of hydrocarbon contamination developed and stabilized at a distance of less than 15 m downgradient from the source area. Exposure of indigenous microoranisms to the contaminant hydrocarbons was evaluated using an array of gene probes targeting common genotypes associated with the aerobic biodegradation of BTEX and naphthalene. Each of the targeted genotypes *alkB*, *nahA*, *nahH*, *todC1C2*, and *xylA* showed significant responses to hydrocarbon exposure. Aerobic mineralization potentials of selected contaminants were greater in sediments collected from within the plume relative to uncontaminated areas, suggesting that an aerobic contaminant-degrading community successfully developed within the plume. An increase in aerobic degradation activity coincided with the arrival of the hydrocarbon front within the well field. The Natural Attenuation Study at Columbus AFB successfully linked adaptations of indigenous microorganisms to hydrocarbon exposure during a transient field study.

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Changes in Subsurface Catabolic Gene Frequencies during Natural Attenuation of Petroleum Hydrocarbons

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Subsurface hydrocarbon contamination associated with petroleum spills is a widespread occurrence that presents a significant threat to groundwater resources. The natural attenuation test site (NATS) at Columbus Air Force Base, MS, provided a unique opportunity to monitor changes in the molecular microbial ecology as well as stimulation of natural biodegradative processes under transient field study conditions. A large, synthetic jet fuel mixture containing BTEX compounds and naphthalene in a decane carrier was introduced into the subsurface. Over 462 days, a plume of hydrocarbon contamination developed and stabilized at a distance of less than 15 m downgradient from the source area. Exposure of indigenous microorganisms to the contaminant hydrocarbons was evaluated using an array of gene probes targeting common genotypes associated with the aerobic biodegradation of BTEX and naphthalene. Each of the targeted genotypes *alkB*, *nahA*, *nahH*, *todC1C2*, and *xylA* showed significant responses to hydrocarbon exposure. Aerobic mineralization potentials of selected contaminants were greater in sediments collected from within the plume relative to uncontaminated areas, suggesting that an aerobic contaminant-degrading community successfully developed within the plume. An increase in aerobic degradation activity coincided with the arrival of the hydrocarbon front within the well field. The Natural Attenuation Study at Columbus AFB successfully linked adaptations of indigenous microorganisms to hydrocarbon exposure during a transient field study.

Introduction

Hydrocarbon contamination of aquifers due to petroleum spills presents a significant threat to groundwater resources in many areas. Once introduced into the subsurface, relatively soluble hydrocarbons, such as the BTEX compounds, can migrate with the groundwater and threaten downgradient water resources. In contaminated areas where no direct threat to public water resources is evident and natural conditions exist for the biodegradation of the target compounds, natural attenuation may be a viable strategy for site remediation. For natural attenuation to become a more generally accepted means of site remediation, a mechanistic understanding of the capacity and biodegradative response of the indigenous subsurface microbial community must be developed.

The ability to monitor changes in microbial populations reflecting contaminant exposure has advanced with the development of molecular based laboratory techniques. Misrepresentation and underestimation of the microorganisms in natural samples mediating natural attenuation can be improved with the application of biochemical assays, such as nucleic acid diagnostics (*1*–*3*). DNA hybridization is currently a powerful diagnostic tool for monitoring microorganisms in diverse environmental samples including soil, sediment, activated sludge, and groundwater. Increased biochemical knowledge has provided the ability to characterize, monitor, and assess the microbiological potential for any given site, without involving any culture bias (*4*). Genotypes commonly associated with the degradation of many environmental contaminants have been evaluated in natural samples (*1*, *5*–*13*). The objective of this investigation was to exploit the growing database on catabolic genes associated with biodegradation to provide a molecular diagnostic framework to document functional natural attenuation at the microbial level.

The research presented in this paper describes the natural attenuation of petroleum hydrocarbons during a controlled, interdisciplinary field-scale natural gradient research experiment, termed the Natural Attenuation (NAT) Study, conducted at Columbus Air Force Base (AFB), MS. These observations directly linked molecular and physiological changes in the microbial community to field observations of contaminant attenuation in a transient system. Previous field experiments at Columbus AFB provided detailed knowledge of the aquifer's hydraulic features (*14*–*19*) as well as the natural biodegradative potential (*20*). After preliminary characterization of the site's microbiology (*12*) and geochemistry, the study aquifer was contaminated with a "model" jet fuel consisting of BTEX, naphthalene, and decane. Hydrocarbon distribution was determined by gas chromatography on groundwater samples. Microbiological data were collected through laboratory analyses of subsurface core samples recovered from contaminated and uncontaminated areas of the study aquifer in order to determine the effects of contaminant exposure on indigenous microorganisms.

Experimental Section

Design of the Natural Attenuation Field Study. The Columbus AFB Groundwater Research Facility supports an expansive array of multilevel groundwater sampling wells that provide analytical points in three-dimensional detail. Since it is widely known that groundwater microbiology does not accurately reflect aquifer sediment microbiology (*21*), a network of borehole sites was designed to collect core samples at strategic locations (Figure 1). Five cores representing depths

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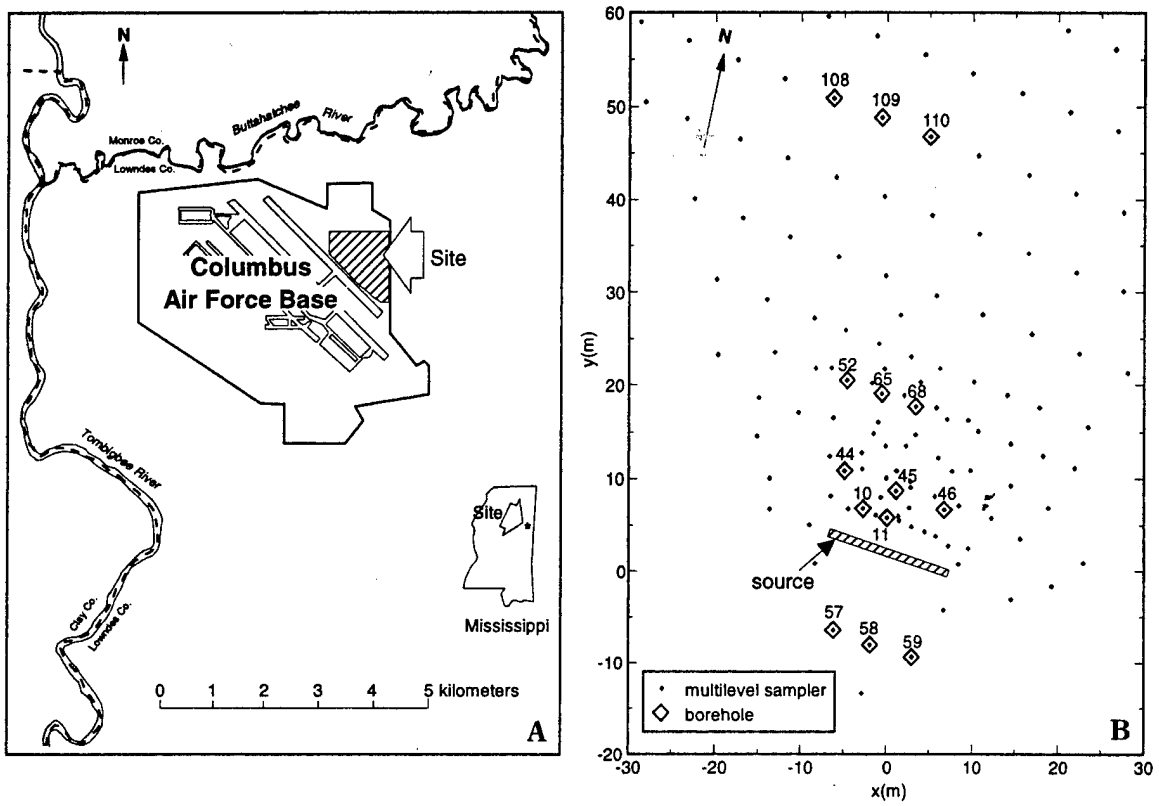

FIGURE 1. Maps of the Columbus AFB Groundwater Research Facility, Columbus, MS. The inset in panel A shows the position of the town of Columbus in the state of Mississippi. Panel A shows the position of the research area on Columbus AFB. Panel B presents a plan view of the research area at Columbus AFB, showing the borehole locations in comparison with the contaminant source trench.

ranging from 4 to 8 m below ground surface were collected from each borehole. Control samples that were not exposed to hydrocarbons during the study were obtained from areas both upgradient and downgradient of the source trench. To relate sediment microbiology to groundwater chemistry, each sample borehole was located near a multilevel groundwater sampling well.

The contaminants chosen for the Natural Attenuation Study represented a model jet fuel, with approximately 25% of the total contaminant mass consisting of BTEX and naphthalene and the remaining 75% consisting of decane. An aqueous solution containing 1 kg of potassium bromide was added to the source mixture to provide a conservative reference tracer for the experiment. The initial bromide concentration in the source material was approximately 40 mg/L. For accurate representation of an actual contaminated site, the contaminants were placed in a trench excavated below the water table. To do this, the unsaturated soil was removed above the water table, the aquifer was locally dewatered, and sheet piling was driven into the subsurface to create a 30-m^3 trench. This trench was filled with the contaminant mixture that had been loaded onto a coarse sand mixture (Table 1). After the trench was filled with the contaminated sand, the sheet piling was removed using a crane, the unsaturated soil was replaced, and the experiment was officially started.

Aquifer sediment samples were recovered prior to contamination of the site (for background analyses) and then 40, 164, 278, and 462 days into the experiment. For the sampling dates at 40 and 164 days, a total of 60 core samples were collected from the field site, while 70 cores were recovered at 278 and 462 days. Samples from the source trench were also collected during each sampling period. In association with the core samples, groundwater samples were collected for geochemical (dissolved oxygen, temperature, pH) and hydrocarbon analyses.

TABLE 1. Compounds Included in the Contaminant Source for the Natural Attenuation Study at Columbus AFB

compound	concn[a]	compound	concn[a]
bromide	45	*p*-xylene	1 134
benzene	7.6	naphthalene	1 282
toluene	1 083	decane	11 713
ethylbenzene	1 163		

[a] Concentration values are given as mg/kg of soil, except for bromide, which is listed as mg/L of groundwater.

Description of the Study Site and Sample Collection. The study site is a shallow unconfined alluvial aquifer averaging approximately 11 m in thickness located on Columbus AFB, Columbus, MS (*14*). Aquifer material ranges from sand (fine granular) to gravelly sand (coarse granular). Background hydrological and microbiological information have been previously described (*12, 14–19*). Subsurface core samples were recovered from locations in close proximity to multilevel groundwater sampling wells and processed on-site using aseptic technique as previously described (*12*). The core samples were stored on ice, and laboratory analyses were initiated within 24 h of collection.

Materials and Methods

Analysis of Hydrocarbon Contaminants in Groundwater. Groundwater samples were collected in standard EPA-certified 40-mL vials, each poisoned with two drops of sodium azide and completely filled so as to not leave any headspace. Hydrocarbon determinations were made following EPA Standard Method 602 for purgeable aromatic compounds using purge-and-trap gas chromatography. High-performance liquid chromatography was used for bromide analyses. The method provided an analytical sensitivity of 0.01 mg/L and a lower detection limit of 0.01 mg/L.

TABLE 2. DNA Probes Used in the Columbus AFB Natural Attenuation Study[a]

probe designation	probe target	compounds targeted
alkB	alkane hydroxylase	decane
nahA	naphthalene dioxygenase	naphthalene
nahH	catechol-2,3-dioxygenase	catechol
todC1	toluene dioxygenase	benzene ethylbenzene toluene *p*-xylene
xylA	xylene monooxygenase	toluene *p*-xylene
16S rDNA universal	small subunit 16S rDNA gene	na[b]

[a] Detailed information on these probes can be found in refs *12* and *31*. [b] Not applicable.

Aerobic Microbial Metabolism of ^{14}C-Labeled Aromatic Substrates. Microbial activity was assessed in triplicate using ^{14}C-labeled substrates (specific activities [mCi/mmol]: benzene, 58.2; toluene, 60.0; naphthalene, 49.8; and phenanthrene, 13.3; all >98% purity; Sigma Chemicals, St. Louis, MO) and were performed as previously described (*12*). Briefly, 2 g of aquifer material was suspended in 1 mL of sterile deionized water in a 40-mL glass vial. An 8-mL glass vial placed inside the 40-mL vial contained 0.5 mL of 0.5 M NaOH and served as a $^{14}CO_2$ trap. Approximately 50 000 dpm of ^{14}C-labeled substrate was added, and the vials were sealed with a Teflon-lined screw cap. Mineralization assays were incubated at 25 °C in the dark on a rotary shaker (100 rpm). Microbial activity was halted by addition of 1 mL of H_2SO_4 (2 N) followed by additional shaking for 2 h at 100 rpm. Production of $^{14}CO_2$ was measured at 24 h by liquid scintillation counting. Killed (negative) controls were established by addition of 1.0 mL of H_2SO_4 (2 N) prior to addition of any radiolabeled substrate.

Molecular Diagnostics of Hydrocarbon-Degrading Microorganisms. DNA was extracted from aquifer samples using the direct lysis method based on ballistic disintegration of microorganisms by bead-mill homogenization as previously described (*12*). After cell lysis, the samples were successively alkaline extracted to recover the released DNA. The aqueous samples were concentrated by 2-propanol precipitations at −20 °C overnight. The precipitants were collected by centrifugation and then dialyzed against 3−5 changes of TE buffer. The samples were phenol and chloroform purified and then concentrated by ethanol precipitation. Final products were suspended in 1 mL of sterile TE buffer and stored at −20 °C until used.

DNA hybridization studies were performed as previously described (*12*). DNA samples were vacuum blotted onto nylon membranes using a slot-blot apparatus (Bio-Rad Life Sciences, Hercules, CA) and fixed by baking for 1 h at 80 °C, rinsed in 2× SSC (20× SSC; g/L: sodium chloride, 17.53; sodium citrate, 8.82; pH 7.0), and then incubated for 1 h at 80 °C. DNA samples were hybridized with the catabolic gene probes listed in Table 2. Radiolabeled single-stranded DNA gene probes were prepared by asymmetric polymerase chain reaction. Radiolabeled 16S rDNA universal oligonucleotide probes were prepared using a 5′ terminus DNA labeling system (Gibco BRL, Gaithersburg, MD). Un-incorporated radionucleotides were removed by using NucTrap Push Columns (Stratagene, LaJolla, CA) and quantified by liquid scintillation counting. DNA values for each hybridization experiment were determined in nanogram amounts as previously described (*12*).

Statistical Analyses and Database Development. MANOVAs and discriminant analyses were performed using the Statistical Package for Social Sciences (SPSS; Jandel Scientific). The Columbus AFB Natural Attenuation Study database was developed by Mike McGee at the Tennessee Valley Authority Engineering Laboratory (Norris, TN) in conjunction with The Center for Environmental Biotechnology at The University of Tennessee.

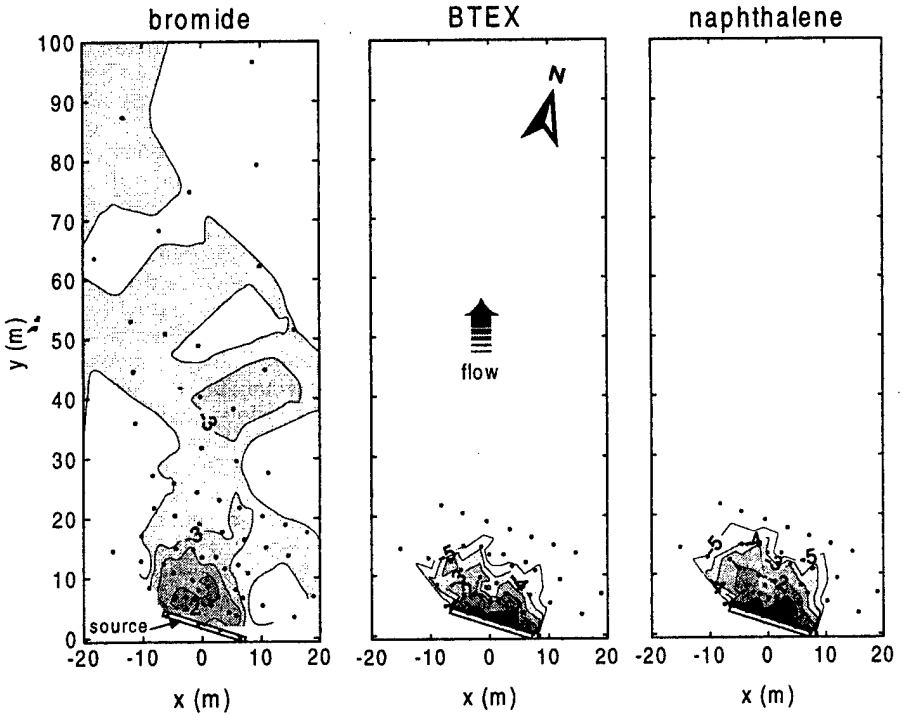

FIGURE 2. Comparison of bromide, total BTEX, and naphthalene aqueous-phase concentrations after 462 days. Concentration data are presented as $\log_{10}$ relative concentrations where vertically averaged concentration measurements from multilevel sampling wells (black dots) have been normalized by initial constituent concentrations.

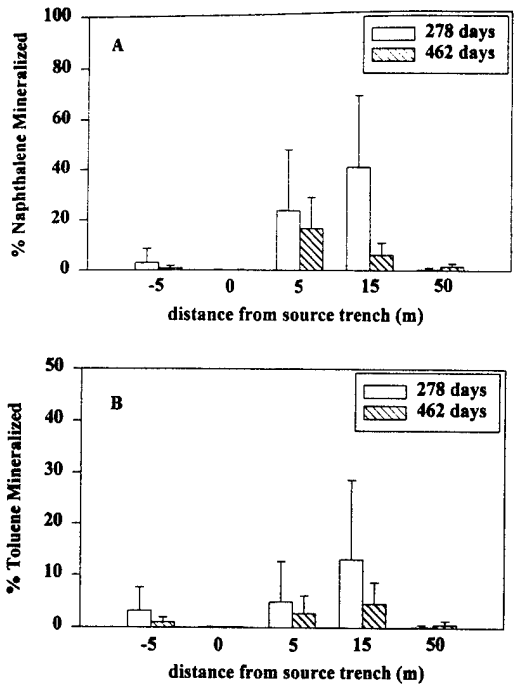

FIGURE 3. Mineralization of naphthalene (A) and toluene (B) during a 24-h snapshot in aquifer samples collected from Columbus AFB at both 278 and 462 days. The upgradient and downgradient values represent the average of 15 individual core samples, the source values represent the average of 7 individual core samples, and both the plume (5 m from the source trench) and plume fringe (15 m from the source trench) values represent the average of 20 individual core samples collected at each time point in the field experiment.

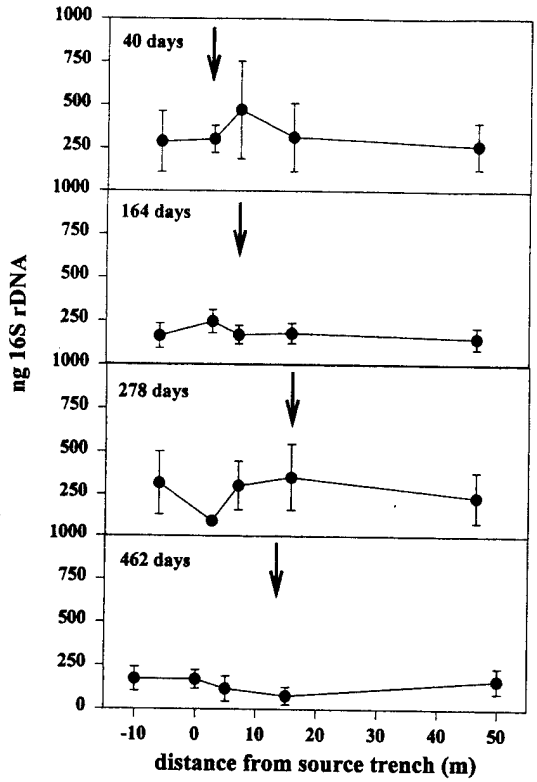

FIGURE 4. Distribution of total biomass, as judged by concentration of 16S rDNA gene sequences per gram of soil, in subsurface aquifer sediments at Columbus AFB, MS. The data points represent the average of 15–20 individual samples collected at each distance from the source trench. The bold arrows represent the distance the front edge of the contaminant plumes has traveled.

Results

Hydrocarbon Transport. The dissolution of NAPL from the source trench and subsequent migration of the aqueous-phase hydrocarbons in groundwater were monitored over a period of 462 days. Figure 2 compares the spatial distribution of the BTEX and naphthalene plumes with that of bromide (nonreactive reference tracer) at 462 days. Concentration measurements for all constituents have been normalized by their initial concentrations and vertically averaged over a common vertical interval of 10 m. Note that decane data are not presented because the extremely limited aqueous solubility of this compound (approximately 6 μg/L) generally precluded detection in groundwater samples. The limited displacement of the BTEX and naphthalene compounds relative to bromide clearly indicates attenuation of the hydrocarbons during the study. The bromide plume exhibited a highly asymmetric concentration distribution in the longitudinal dimension. The maximum bromide concentration occurred approximately 5 m downgradient of the source trench with the advancing side of the plume extending downgradient more than 100 m and the trailing side of the plume extending upgradient only a few meters. Conversely, the hydrocarbon plumes show monotonically decreasing concentrations downgradient of the source with the plume fronts advancing less than 15 m from the source.

Mineralization of Target Contaminants. Aerobic mineralization assays of contaminant hydrocarbons (24 h duration) revealed an increased ability to oxidize contaminant substrates in sediments exposed to hydrocarbon contamination. Naphthalene was aerobically mineralized to a greater extent than toluene. At 278 days, the greatest naphthalene mineralization potentials were observed in samples collected from the plume fringe (Figure 3A). Mineralization of naphthalene in these samples averaged greater than 40% over 24 h but ranged as high as 60% in some samples. Enhanced mineralization of naphthalene was also seen in samples recovered from within the plume. Average naphthalene mineralization rates for plume samples were 21% within 24 h but ranged as high as 42% in some samples. These enhanced rates of naphthalene mineralization were sustained through 462 days of the study (Figure 3A). The highest rates of naphthalene mineralization at 462 days were observed in samples collected from the plume area, with the average mineralization value being 20% and the maximum value being 35% within 24 h. Toluene mineralization followed a similar trend at 278 days, with the largest rates of mineralization observed in samples collected along the plume fringe (Figure 3B). Toluene mineralization averaged 10% along the fringe, with values ranging as high as 32% within 24 h. Lower rates of toluene mineralization were observed after 462 days (Figure 3B), with mineralization rates of 10% and 12% in the plume and plume fringe samples, respectively. In contrast, mineralization of toluene and naphthalene was less than 5% in uncontaminanted samples collected from sites both upgradient and downgradient of the hydrocarbon plume at both 278 and 462 days.

Catabolic Gene Dynamics. Changes in microbial community structure in response to hydrocarbon exposure and degradation were evaluated using an array of DNA probes (Table 2) designed to target specific genes encoding enzymes commonly associated with the biodegradation of the compounds listed in Table 1. While not an exhaustive list of potential DNA probes, these genes were previously demonstrated to be widely distributed in aquifer samples collected from Columbus AFB (*12*). The parameter percent community was devised to evaluate the response of a given genotype as a function of the entire microbial community (*3*).

Estimates for total microbial biomass were calculated based on hybridization of whole microbial community DNA

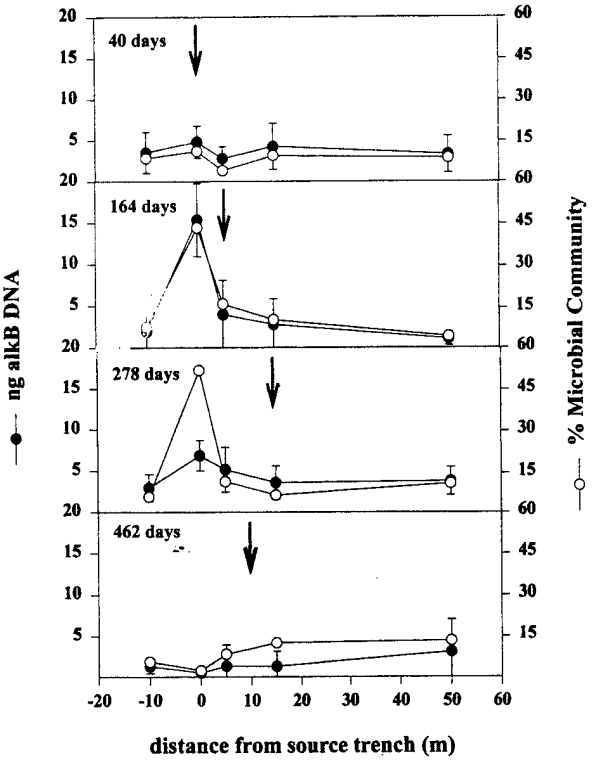

FIGURE 5. Distribution of decane-degrading genotypes, as judged by concentration of *alkB* gene sequences per gram of soil, in subsurface aquifer sediments at Columbus AFB, MS. The data points represent the average of 15–20 individual samples collected at each distance from the source trench. The bold arrows represent the distance the front edge of the contaminant plumes has traveled.

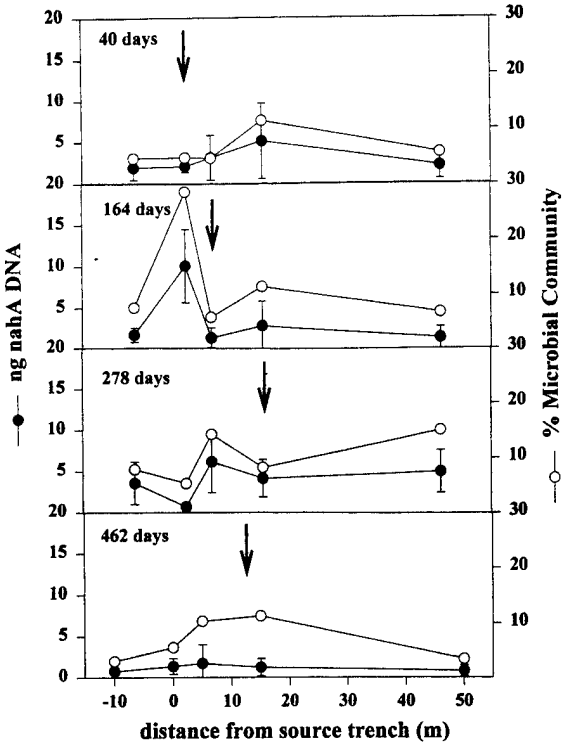

FIGURE 6. Distribution of naphthalene-degrading genotypes, as judged by concentration of *nahA* gene sequences per gram of soil, in subsurface aquifer sediments at Columbus AFB, MS. The data points represent the average of 15–20 individual samples collected at each distance from the source trench. The bold arrows represent the distance the front edge of the contaminant plumes has traveled.

with a DNA probe targeting the 16S rDNA gene. The greatest impact on microbial biomass at the study site was seen in the source trench (Figure 4). The abundance of 16S rDNA genes significantly increased at 164 days ($p < 0.05$) and then significantly decreased ($p < 0.001$) at 278 days.

Microorganisms possessing the *alkB* genotype were also impacted the greatest in the source trench (Figure 5). This was predicted based on the finding that decane did not leach from the source trench in detectable concentrations. *alkB* genotypes were determined to be at significantly higher abundances ($p < 0.001$) in the source trench at 164 and 278 days, with the greatest enrichment occurring between 40 and 164 days, where *alkB* target sequences increased from just under 5 to over 15 ng/g of soil. Alkane-degrading genotypes steadily increased between 40 and 278 days, with this population starting at 11% at 40 days, increasing to 44% at 278 days, and peaking at 52% of the total microbial community at 462 days.

Naphthalene impacted the microbial community of the source trench as well as that present downgradient of the source trench (Figure 6). Between 40 and 164 days, *nahA* genotypes present in the source trench increased from 2 to 10 ng/g of soil, a 5-fold enrichment ($p < 0.001$). However, at 278 days, the impact of naphthalene moved downgradient of the source where *nahA* genotypes were found at significantly elevated levels ($p < 0.001$). The *nahA* genotypes subsequently suffered a population crash at 278 days in the source material where abundances decreased 14-fold ($p < 0.001$). However, an enrichment for *nahA* genotypes in the plume area coincided with the observed decrease in the source. As a function of the total microbial community, the naphthalene-degrading population increased from just under 5% at 40 days to more than 29% at 164 days, followed by a decrease to 5% at both 278 and 462 days. In the plume area,

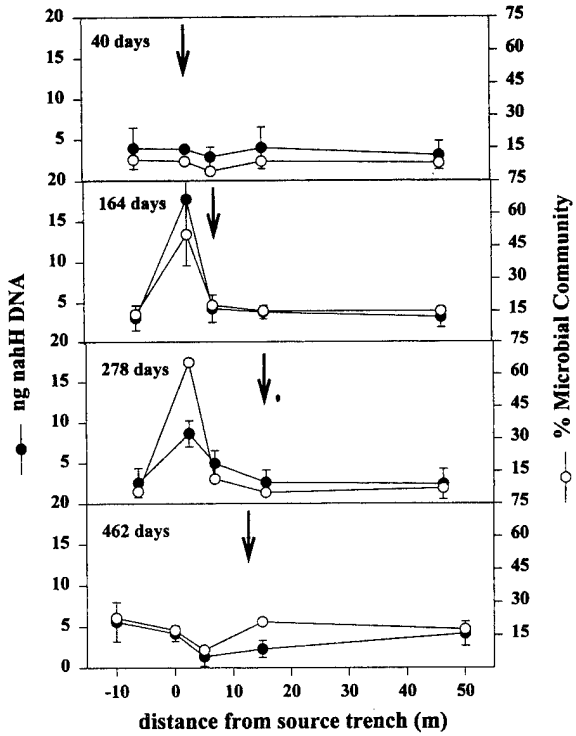

FIGURE 7. Distribution of catechol-degrading genotypes, as judged by concentration of *nahH* gene sequences per gram of soil, in subsurface aquifer sediments at Columbus AFB, MS. The data points represent the average of 15–20 individual samples collected at each distance from the source trench. The bold arrows represent the distance the front edge of the contaminant plumes has traveled.

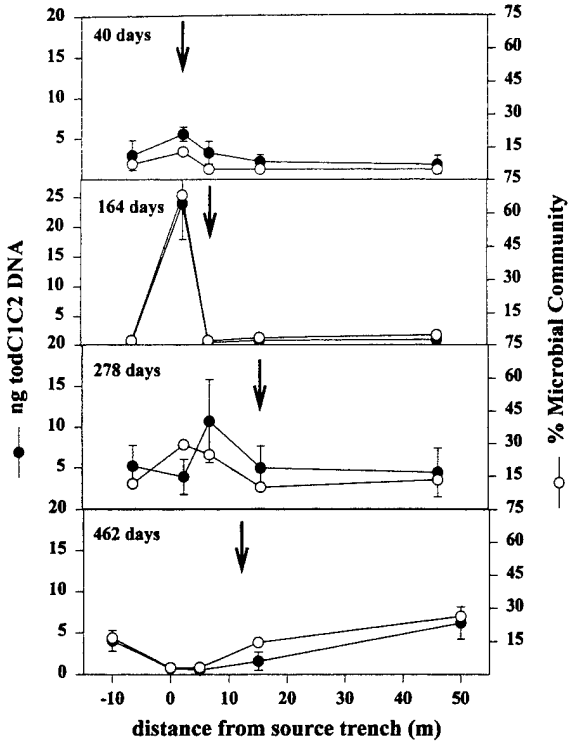

FIGURE 8. Distribution of aromatic hydrocarbon-degrading genotypes, as judged by concentration of *todC1C2* gene sequences per gram of soil, in subsurface aquifer sediments at Columbus AFB, MS. The data points represent the average of 15–20 individual samples collected at each distance from the source trench. The bold arrows represent the distance the front edge of the contaminant plumes has traveled.

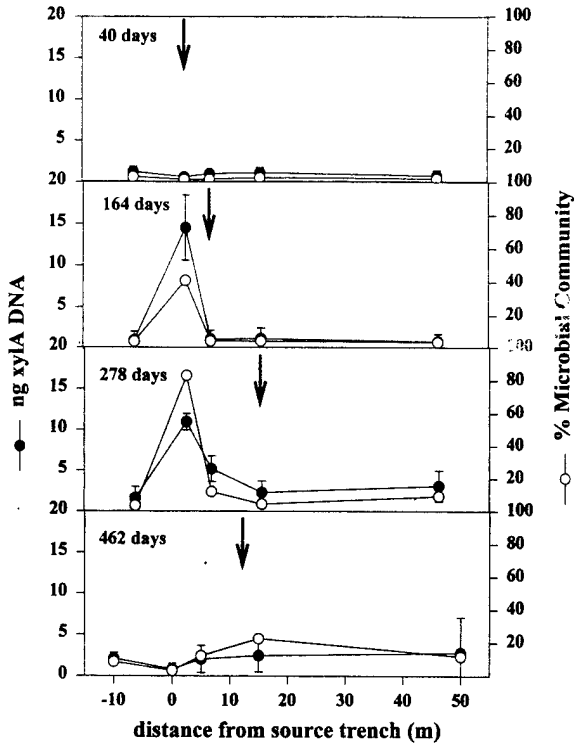

FIGURE 9. Distribution of aromatic hydrocarbon-degrading genotypes, as judged by concentration of *xylA* gene sequences per gram of soil, in subsurface aquifer sediments at Columbus AFB, MS. The data points represent the average of 15–20 individual samples collected at each distance from the source trench. The bold arrows represent the distance the front edge of the contaminant plumes has traveled.

nahA genotypes increased from 6 to 14% of the total microbial community.

Figure 7 shows the response of *nahH* genotypes to hydrocarbon exposure overtime at Columbus AFB. In the source trench, *nahH* genotypes increased 4.5-fold to 18 ng/g of soil ($p < 0.001$). Elevated levels of *nahH* genotypes (9 ng/g of soil) were also seen in samples from the plume area at 278 days. These elevated levels of *nahH* genotypes increased from 9% at 40 days to 50% at 164 days and then peaking at 66% of the microbial community at 278 days.

Toluene-degrading organisms were greatly impacted by migration of the hydrocarbons in the Columbus AFB aquifer. *todC1C2* genotypes showed a significant response ($p < 0.001$) to the aromatic contaminants in the source trench at 40 days, which was sustained through 164 days (Figure 8). At 278 days, the abundance of *todC1C2* genotypes decreased 6-fold in the source trench but correspondingly increased 17-fold in the plume region ($p < 0.001$). At 462 days, *todC1C2* genotype abundance was significantly lower in both the source trench and the plume region ($p < 0.001$). Toluene-degrading genotypes increased from 13% at 40 days to 68% at 164 days of the microbial community in the source trench. At 278 days, *todC1C2* genotypes were elevated in both the source trench and the plume area, representing 29% and 25% of the microbial community.

The genotype in the lowest abundance prior to hydrocarbon exposure was *xylA* (Figure 9). *xylA* genotypes throughout the Columbus AFB aquifer remained below 1 ng at 40 days. However, at 164 days, *xylA* genotypes increased 26-fold to 15 ng/g of soil in the source trench ($p < 0.001$). This significantly higher level of *xylA* genotypes was sustained at 11 ng/g of soil through 278 days ($p < 0.001$). At 462 days, a 13-fold decrease in *xylA* genotypes was seen in the source trench. *xylA* genotypes increased from 1% of the microbial community to 41% at 164 days before peaking at 83% at 278 days.

Discussion

The Natural Attenuation Study at Columbus AFB was designed to directly link changes in microbial community structure to not only the attenuation of migrating hydrocarbons in groundwater but also the elimination of contaminant mass from the aqueous phase. Data from previous field investigations, as well as laboratory experiments using aquifer material collected from the study site, demonstrated a potential for the natural attenuation of hydrocarbons within the Columbus aquifer (*12, 14–19*). Subsequent samples collected from the NAT study site successfully linked changes in microbial metabolic activity and community structure with exposure to hydrocarbons.

Changes in microbial community structure in response to hydrocarbon contamination was monitored over time using an array of DNA probes targeting specific genotypes associated with the degradation of contaminant hydrocarbons. These shifts in microbial community structure directly reflect the migration of contaminants in groundwater (Figures 3, 10, and 11). At 40 days, little contaminant was observed outside of the source trench, and the only significant enrichment for degradative microorganisms at 40 days was seen for *todC1C2* genotype. At 164 days, every degradative genotype significantly increased in samples collected from the source. Aquifer samples recovered from the area approximately 5 m downgradient from the source trench prior to hydrocarbon exposure showed aerobic mineralization levels of under 10% within 24 h for both toluene and naphthalene. Similar activity trends were seen at 164 days.

Both *todC1C2* and *nahA* genotypes reached peak population levels in the source trench at 164 days but underwent

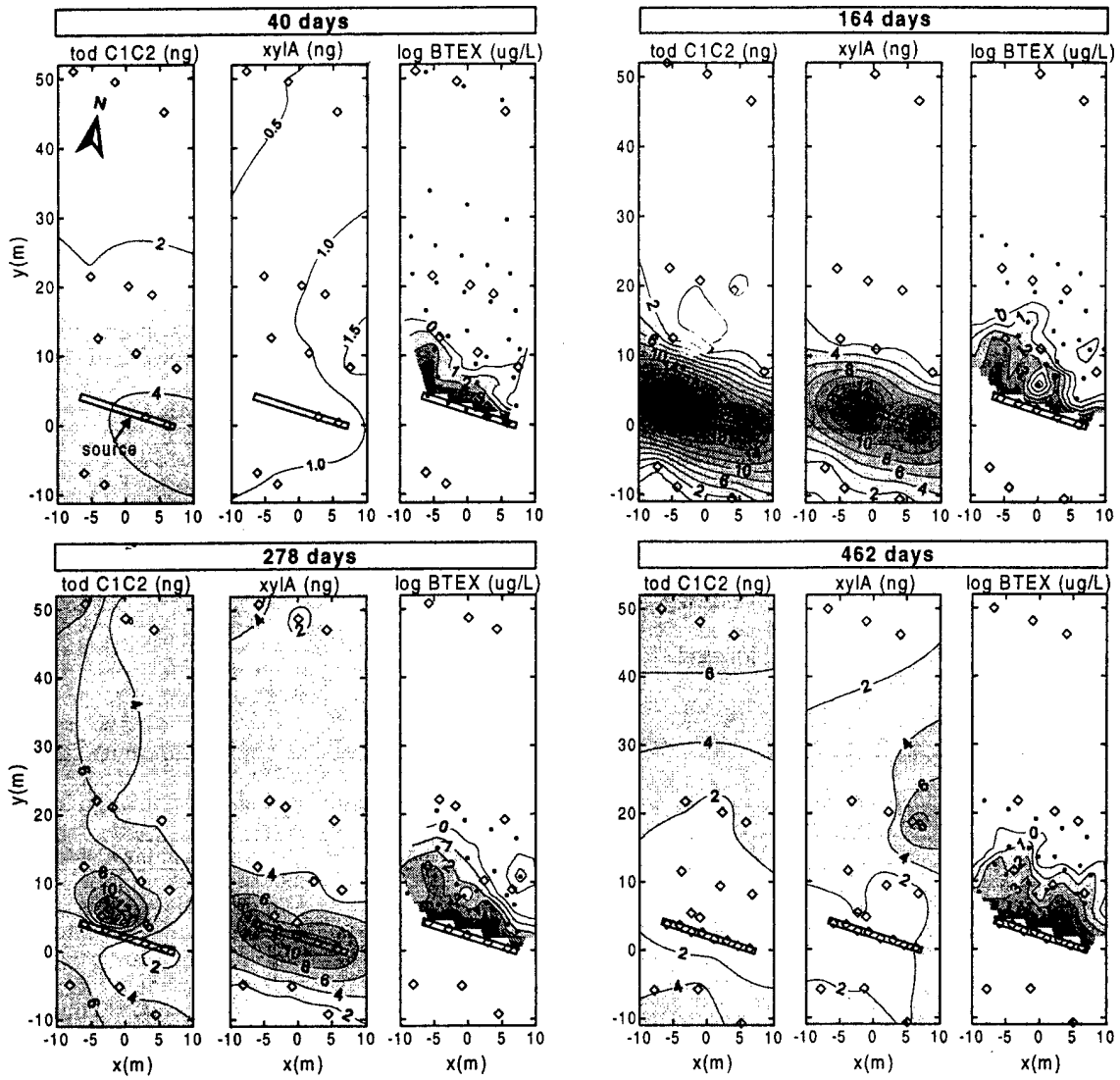

FIGURE 10. Two-dimensional representation of the cause and effect relationship between migration of aromatic BTEX compounds downgradient of the contaminant trench and aromatic hydrocarbon-degrading genes (*todC1C2* and *xylA*). Both BTEX and individual gene frequency measurements have been vertically averaged. Note that diamond symbols represent biological soil sampling sites, whereas black dots denote multilevel sampling wells.

significant decreases at 278 days. The population crash of these genotypes and the migration of hydrocarbons downgradient of the source trench initiated changes in the community structure. At 278 days, *nahA* and *todC1C2* genotypes as well as drastic increases in microbial metabolism of toluene and naphthalene were observed at elevated levels downgradient of the source trench. It appears that the initial arrival of organic contaminants in the plume area stimulated a rapid increase in microbial mineralization of hydrocarbons, as compared with both data from previous sampling periods at 40 and 164 days and uncontaminated samples from the same sampling trip. The increase in mineralization activity along the plume fringe is most likely due to either the release of metabolic byproducts from microorganisms in the plume area or the presence of hydrocarbons below detection limits, or both. At 462 days, stabilization of both the plume front and degradative genotypes was observed. The elevated levels of microbial metabolism in samples from the plume fringe at 278 days were not sustained through 462 days of the field experiment. However, elevated levels of aerobic mineralization observed at 462 days suggest that conditions, especially dissolved oxygen levels, were not limiting to these mechanisms of biodegradation. At 462 days, increased levels of the degradative genotypes *nahA* and *todC1C2* were present in downgradient core samples. This is most likely due to either the presence of hydrocarbons below detectable limits or the release of metabolic byproducts from microorganisms located in the plume and along the plume fringe that stimulated bacteria populations further downgradient.

Analysis of cores collected periodically from the source material indicated gradual decreases in source hydrocarbon concentrations during the study, but compete depletion was not observed for any of the contaminants. For example, source hydrocarbon mass at the 830 days, expressed as percent of the original introduced mass, was 33% for benzene and ranged from 59 to 67% for ethylbenzene, toluene, *p*-xylene, and naphthalene. The stable APM levels observed benzene, toluene, and naphthalene during the last half of the study, in conjunction with the spatial stability of the hydrocarbon plumes, indicating that these compounds were naturally attenuated at rates approximately equal to their mass transfer rates. The observed stability of the ethylbenzene and *p*-xylene plume fronts during the latter stages of the experiment also suggests natural attenuation of these compounds, despite the increasing APM trends throughout the experiment.

Previous field experiments have concentrated on the fate of very dilute aqueous phase injections of contaminants in

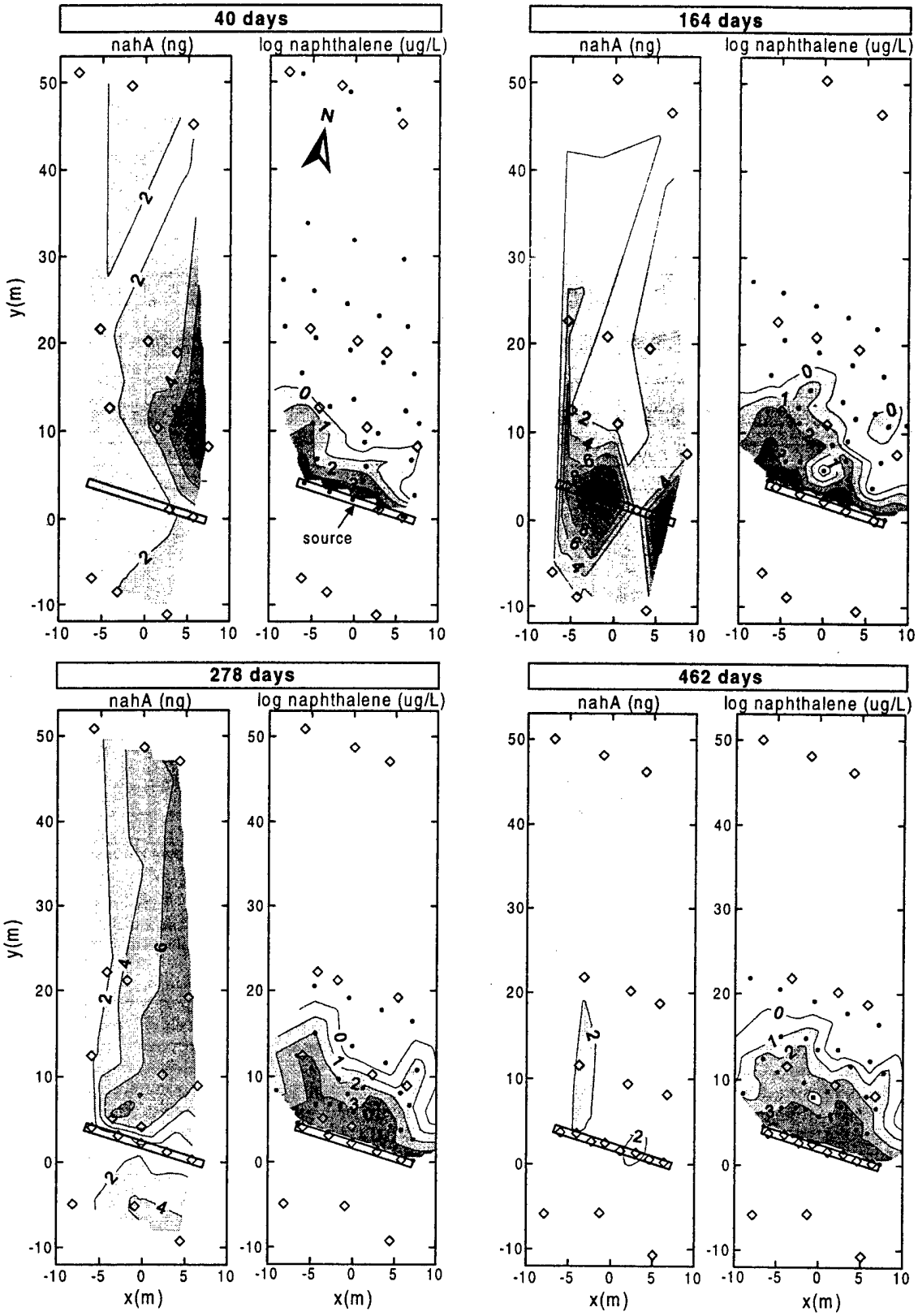

FIGURE 11. Two-dimensional representation of the cause and effect relationship between migration of naphthalene downgradient of the contaminant trench and *nahA* gene frequencies. Both naphthalene and *nahA* measurements have been vertically averaged. Note that diamond symbols represent biological soil sampling sites, whereas black dots denote multilevel sampling wells.

highly localized, strictly hydraulically controlled test plots, areas of widespread and long-term contamination, such as defunct manufactured gas plant sites (*8, 22–24*) or areas associated with fuel spills (*25, 26*). Barker et al. (*27*) showed that dissolved oxygen was the limiting factor to the natural attenuation of dilute BTEX compounds in a sandy aquifer. Madsen et al. (*22*) showed microbial adaptation to organic pollutants at a buried coal tar site with ^{14}C-labeled compound mineralization assays. Davis et al. (*28*) showed that the influences of advection and adsorption on benzene migration

within groundwater could not account for the observed plume attenuation at a manufacturing facility on the eastern seaboard.

Molecular investigations into the microbial ecology of contaminated sites have also primarily explored areas of long-term or chronic pollution. Sanseverino et al. (*8*) showed an elevated level of *nahA* genotypes in soils collected from defunct manufacturing gas plants (MGP) suffering from chronic polyaromatic hydrocarbon contamination, while an increased expression of naphthalene dioxygenase genes was correlated to both *nahA* gene abundance and levels of total PAH in MGP soils (*29*). Recovery of *nahA* genotypes was also described by Herrick et al. (*7*) at other coal tar-contaminated sites. Supplemental work on these sites demonstrated the in situ expression of naphthalene dioxygenase genes in groundwater samples (*13*). The concept of describing microbial communities in mixed-chemical contaminated samples with a multiple gene probe array has only recently been approached (*3, 9, 12, 30*). Hosein et al. (*30*) described changes in the degradative genotypes *xylE* (catechol dioxygenase) and *ndoB* (naphthalene dioxygenase) in bacteria cultured from column experiments. Guo et al. (*9*) described changes in degradative genotypes at a fuel oil-contaminated site and in laboratory microcosms based on the enrichment of several catabolic genes (*xylE, nahAcd, todC1C2BA, tmoABCDE,* and *alkB*). This is currently the only report on the transient responses of indigenous microorganisms to hydrocarbon exposure at the field scale.

The NAT Study at Columbus AFB, MS, was designed as a multidisciplinary field experiment to work toward a better understanding of the fate of petroleum hydrocarbons in groundwater aquifers. The NAT study was initiated as an extension of previous groundwater research at Columbus AFB investigating the influence of macrodispersion on solute transport. During previous studies, it was recognized that understanding microbial interactions with groundwater contaminants was important for accurate delineation of plume migration and management in the subsurface. The investigation of young, transient hydrocarbon plumes that have not yet chemically and biologically weathered present an important foundation for remediation of contaminated groundwater. While physical and chemical processes, including adsorption, dispersion, and diffusion, have been shown to influence the fate and the transport of contaminants in groundwater, microbiological processes work to directly destroy or transform the compounds to minimize associated health risks. The metabolic processes driving the contaminant destruction can often be measured using laboratory analyses, but these results must be interpreted within the context of data collected in the field. Loss of contaminant mass must be documented with field observations in order to infer the natural degradation of contaminants. During the NAT study, we successfully linked changes in specific degradative populations of microorganisms using molecular techniques with laboratory-based measurements of hydrocarbon mineralization along with observations of not only plume attenuation but also substantial mass loss of contaminant within the aquifer.

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